Vac1p coordinates Rab and phosphatidylinositol 3-kinase signaling in Vps45p-dependent vesicle docking/fusion at the endosome

Michael R. Peterson, Christopher G. Burd* and Scott D. Emr

The vacuolar protein sorting (VPS) pathway of *Saccharomyces cerevisiae* mediates transport of vacuolar protein precursors from the late Golgi to the lysosome-like vacuole. Sorting of some vacuolar proteins occurs via a prevacuolar endosomal compartment and mutations in a subset of VPS genes (the class D *VPS* genes) interfere with the Golgi-to-endosome transport step. Several of the encoded proteins, including Pep12p/Vps6p (an endosomal target (t) SNARE) and Vps45p (a Sec1p homologue), bind each other directly [1]. Another of these proteins, Vac1p/Pep7p/Vps19p, associates with Pep12p and binds phosphatidylinositol 3-phosphate (PI(3)P), the product of the Vps34 phosphatidylinositol 3-kinase (PI 3-kinase) [1,2]. Here, we demonstrate that Vac1p genetically and physically interacts with the activated, GTP-bound form of Vps21p, a Rab GTPase that functions in Golgi-to-endosome transport, and with Vps45p. These results implicate Vac1p as an effector of Vps21p and as a novel Sec1p-family-binding protein. We suggest that Vac1p functions as a multivalent adaptor protein that ensures the high fidelity of vesicle docking and fusion by integrating both phosphoinositide (Vps34p) and GTPase (Vps21p) signals, which are essential for Pep12p- and Vps45p-dependent targeting of Golgi-derived vesicles to the prevacuolar endosome.

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Results and discussion

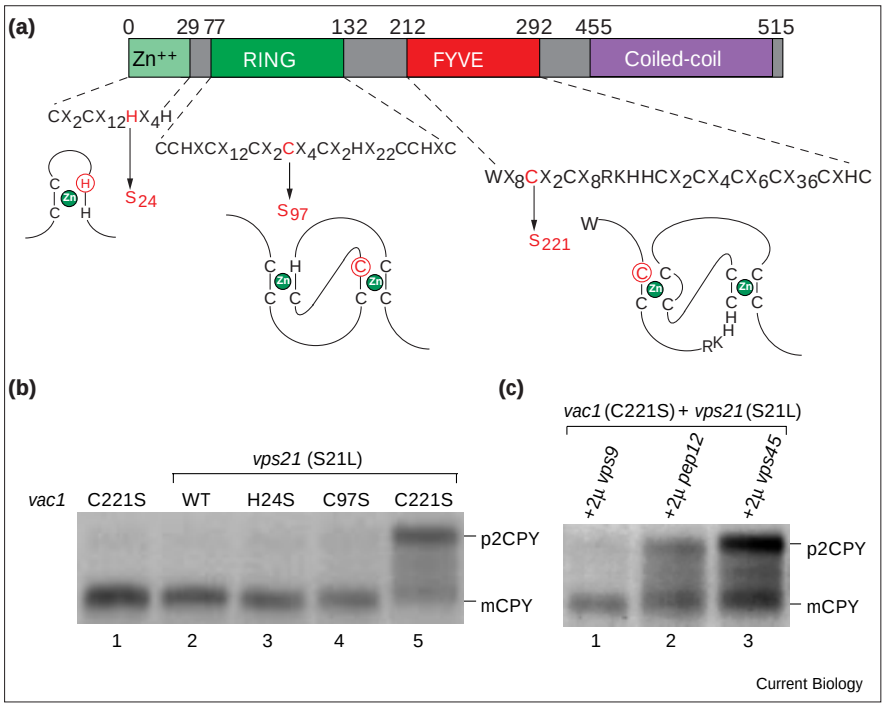
Genetic interactions between *VAC1*, *VPS21* and *VPS9*

Vac1p is a peripheral membrane protein associated with endosomal compartments. It contains a zinc-finger motif (amino acids 8–29) and two zinc-coordinating RING domains (amino acids 77–132 and 212–292). The second RING domain is a member of the FYVE subfamily and has been shown to bind to PI(3)P directly [1,2]. We have shown previously that mutations in *VAC1* that change zinc-coordinating residues of the FYVE domain result in the missorting of vacuolar proteins and temperature-sensitive growth defects (Figure 1a) [1]. These defects can be suppressed by the overexpression of either *VPS45* or *PEP12*, suggesting a close link between Vac1p function and the endosomal SNARE complex responsible for the docking and fusion of Golgi-derived transport vesicles [1,3]. *VPS34* encodes the only PI 3-kinase as yet identified in the yeast genome; its product, PI(3)P, is essential for the VPS pathway [4]. Because FYVE domains function as modular PI(3)P-binding domains, these results suggest that Vac1p links the PI 3-kinase signaling pathway to the Pep12p-mediated fusion of transport vesicles with the prevacuolar endosome [2]. This transport step is also regulated by Vps21p [5], a Rab GTPase closely related to human Rab5. A putative mammalian Vac1p orthologue required for homotypic endosomal fusion [6], early endosomal antigen 1 (EEA1), contains a FYVE domain and has been implicated as an effector of Rab5 on the basis of the observation that it binds Rab5 through a small region immediately preceding its FYVE domain [7]. We therefore explored potential genetic interactions between *VAC1* and *VPS21*.

We introduced single-copy vectors encoding mutant *vps21* alleles into strains with conditional mutations in *PEP12* and *VAC1* to look for potential genetic interactions between these mutations. To assay the VPS pathway in these cells, we performed pulse–chase carboxypeptidase Y (CPY) sorting assays. We noted a severe growth defect and vacuolar protein missorting in the *vac1* (C221S) cells expressing an inactive form of Vps21p that is predicted to be unable to bind GTP — *vps21* (S21L). As can be seen in Figure 1b, *vac1* (C221S) cells expressing the *vps21* (S21L) mutant gene exhibited a severe missorting defect at 26°C, whereas cells expressing *vac1* (C221S) alone at this temperature did not. Expression of *vps21* (S21L) in wild-type cells, or in cells expressing other *vac1* mutants (H24S and CP97S), had little to no effect on CPY sorting. The presence of a synthetic CPY missorting defect in cells expressing *vps21* (S21L) and *vac1* (C221S) indicates that these proteins may act at the same step in the VPS pathway.

Given that the strain utilized in these experiments expresses wild-type Vps21p, these results suggest that Vps21p (S21L) could be binding and inactivating a factor required for Vac1p function. We therefore tested whether overexpression of other class D *VPS* genes or *GDI*/*SEC19*,

Figure 1



Genetic interactions between *VAC1*, *VPS21* and *VPS9*. (**a**) The domains of Vac1p and the mutants utilized in this study: H24S, C97S and C221S [1]. (**b**) Strains expressing *VAC1* (WT) or the indicated *vac1* mutants were transformed with a CEN expression vector (pRS415) encoding *vps21* (S21L). CPY sorting was analyzed by pulse–chase analysis [15] and immunoprecipitation of CPY [16]. The conversion of Golgi-modified precursor CPY (p2CPY) to mature CPY (mCPY) indicates delivery of CPY to the vacuole, whereas accumulation of p2CPY, as is observed in *vac1* mutants [1], indicates a block in the VPS pathway. Co-expression of *vac1* (C221S) and *vps21* (S21L) results in a synthetic vacuolar missorting phenotype (lane 5). (**c**) Cells expressing *vac1* (C221S) and *vps21* (S21L) were transformed with multicopy 2μ vectors (pRS423) encoding Vps9p, Pep12p or Vps45p and CPY sorting was analyzed by pulse–chase analysis. Vps9p completely rescues the sorting defect (lane 1), whereas Pep12p only partially rescues the sorting defect (lane 2).

which encodes a Rab guanine-nucleotide dissociation inhibitor (Rab GDI), could suppress the growth defect and *vps* phenotype of this strain. Overexpression of *VPS9* caused a nearly complete suppression of the *vps* defect, whereas overexpression of *PEP12* had only a small effect (Figure 1b); *VPS45* and *SEC19* had little to no effect (Figure 1b and data not shown). This suggests that Vps9p is the factor titrated out by expression of GDP-locked Vps21p. Because Vps9p has been shown to be a guanine-nucleotide exchange factor (GEF) for Vps21p [8], these results raise the possibility that Vac1p may interact with GTP-loaded Vps21p.

Activated Vps21p binds Vac1p

Vps45p, Vac1p and Sec18p/N-ethylmaleimide-sensitive factor (NSF) have all been shown to bind a Pep12p–glutathione-S-transferase (GST) fusion protein [1]. To further demonstrate interactions using directed yeast two-hybrid experiments, three 'prey' *VPS21* constructs were created using wild-type *VPS21*, *vps21* (Q66L) or *vps21* (S21L). As a control for Rab specificity, a prey construct that contained a GTPase-deficient *ypt7* gene, *ypt7* (Q68L), was made. A 'bait' construct encoding full-length Vac1p was created and tested for binding to Vps21p. We found that Vac1p interacted with wild-type Vps21p and the GTPase-deficient Vps21p (Q66L) mutant protein, but not with the GDP-locked Vps21p (S21L) mutant protein (Table 1). Interestingly, we found that Vps21 (Q66L) was also capable of binding the mammalian Vac1p orthologue EEA1 with conformational specificity, suggesting a high level of conservation in the binding domain(s) (data not shown). These results indicate that Vac1p may function as a downstream effector of Vps21p.

To test which domains of Vac1p bind Vps21p, we used a set of *vac1* mutants in which we made substitutions in zinc-binding cysteine or histidine residues of the zinc-finger domain (H24S), the first RING domain (C97S), or the FYVE domain (C221S; Figure 1a) [1]. Each of these mutant *vac1* genes was cloned into the bait vector and assayed for interactions with the *vps21* (Q66L) two-hybrid prey construct. We found that a mutation in the FYVE domain of *vac1* (C221S), abrogated interaction with Vps21p (Q66L), indicating that this domain may play some role in mediating this interaction. A truncated *VAC1* bait construct encoding only the zinc-binding domains (amio acids 1–325) was sufficient to mediate a weak interaction with *vps21* (Q66L), indicating that the carboxy-terminal portion of Vac1p may enhance binding to Vps21p.

To confirm our two-hybrid results and ascertain their biological significance, co-precipitation experiments were used as an independent test for association of Vac1p and Vps21p. We fused the *Staphylococcus aureus* Protein-A domain in-frame to Vps21p mutants that were either GTPase-deficient (Q66L) or unable to bind GTP (S21L), and introduced these constructs into a wild-type strain (SEY6210). Binding of Vac1p to the fusion proteins was

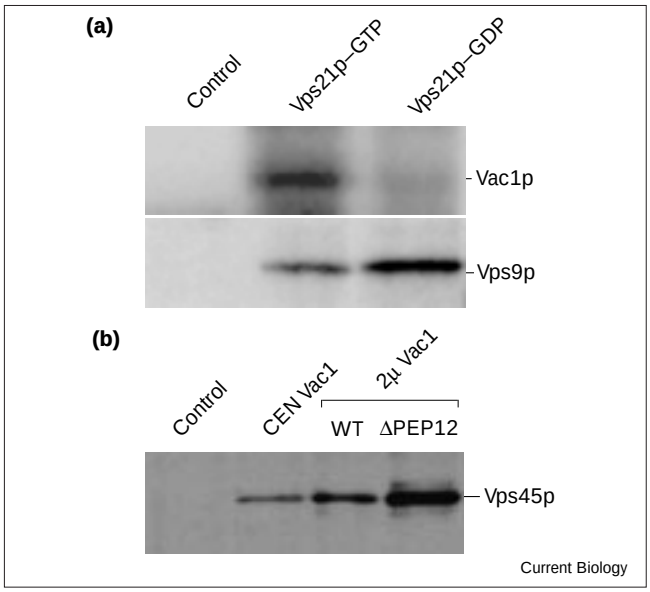
Table 1

The protein–protein interactions of Vac1p in yeast two-hybrid experiments.

	Bait:				
	VAC1 (WT)	*VAC1* (1–325)	*vac1* (H24S)	*vac1* (C97S)	*vac1* (C221S)
Prey:					
vps21 (Q66L)	++	–	++	++	–
VPS21 (WT)	+	–	+	+	–
vps21 (S21L)	–	–	–	–	–
ypt7 (Q68L)	–	–	–	–	–
VPS45	+++	+++	+++	+++	+
VPS9	–	–	–	–	–

The indicated bait and prey constructs were transformed into the reporter strain HF7c (Clontech), and transformants were plated on minimal media lacking histidine and containing 1 mM 3-amino-2,4-triazole. Growth on this media indicates interaction between bait and prey. After several days at 30°C, plates were scored for growth and placed into four groups, from +++ to –, depending on growth rate. Vac1p binds GTP-bound Vps21p. *VAC1* (1–325) represents a truncated version of *VAC1* that encompasses only the zinc-binding domains.

Figure 2



(a) Vps21p binds Vac1p and Vps9p. Fusion constructs of Protein A and GTPase-deficient Vps21p (Q66L) or GDP-locked Vps21p (S21L) bound preferentially to Vac1p and Vps9p, respectively. Vps9p is enriched approximately fivefold when co-precipitated with GDP-locked Vps21p (Vps21p–GDP) rather than with GTPase-deficient Vps21p (Vps21p–GTP). (b) Vps45p binds Vac1p. A fusion protein consisting of full-length Vac1p containing two Protein-A domains precipitates Vps45p at both CEN and multicopy 2μ levels (CEN Vac1 and 2μ Vac1, respectively). As a control, the multicopy 2μ *VAC1*::Protein A construct (2μ Vac1) was transformed into *PEP12Δ::HIS3* (ΔPEP12) cells, confirming that the Vac1p–Vps45p interaction is not due to bridging by Pep12p. The protocol for these co-precipitation experiments is described in [17] and is briefly as follows: mid-log phase cultures were converted to spheroplasts and lysed. The cleared lysates were solubilized with digitonin and chromatographed on IgG–sepharose columns. The columns were washed and bound proteins were eluted with acetic acid. The eluates were subjected to SDS–PAGE and immunoblotting with appropriate antisera.

assayed by chromatography on IgG–sepharose columns, and western blotting of the resulting eluates with polyclonal antisera. As expected from the two-hybrid interaction analysis, GTP-bound Vps21p (Q66L) co-precipitated Vac1p, whereas GDP-bound Vps21p (S21L) did not (Figure 2a). As a control, we also probed for Vps9p, a Vps21p GEF, which, as predicted in the genetic experiments in Figure 1b, binds preferentially to the GDP-locked form of Vps21p (Figure 2a). Together with the results of the two-hybrid assays, these experiments indicate that the activated GTP-bound form of Vps21p binds directly to Vac1p, implicating a role for Vac1p as an effector of this Rab GTPase.

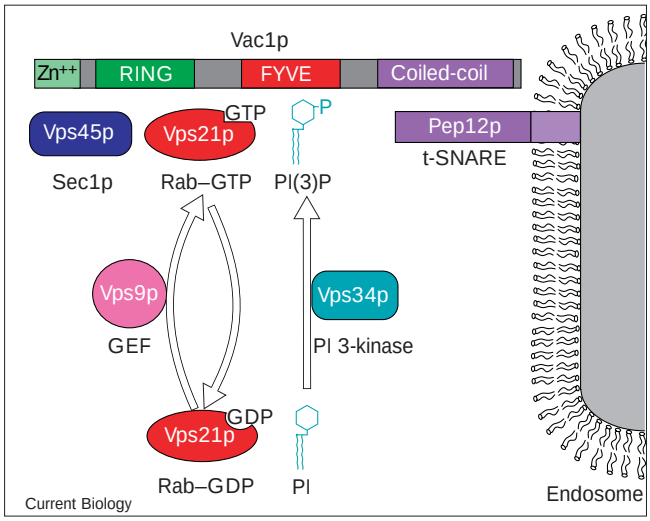
Vac1p interacts with the Sec1p homologue Vps45p

The Sec1p homologue Vps45p has been shown to be a potent suppressor of the CPY-sorting and growth defects of *vac1* mutants, suggesting that Vac1p and Vps45p function at the same step of the VPS pathway [1,3]. We therefore tested whether Vac1p and Vps45p interact physically. We created a full-length *VPS45* prey construct and found that it bound the *VAC1* bait construct (Table 1). In addition, the *vac1* (C221S) FYVE mutant partially abrogated this interaction, suggesting that this domain may play a role in the association. Truncated Vac1p (amino acids 1–325) still bound Vps45p, indicating that the binding site for Vps45p is in the amino-terminal half of Vac1p.

To confirm our two-hybrid results, we again performed co-precipitation experiments to demonstrate specific binding between Vac1p and Vps45p. A *VAC1*–Protein A fusion was constructed in yeast expression vectors and transformed into wild-type cells. As shown in Figure 2b, Vps45p is precipitated by the Vac1p–Protein A fusion at both CEN and multicopy 2μ levels. As a control, to ensure that the Vac1p–Vps45p interaction is direct, we performed this experiment in the *pep12*Δ::*HIS3* (CBY31) strain and found that the Vac1p–Protein A fusion was still capable of precipitating Vps45p; indeed, the levels of Vps45p were increased several-fold relative to wild-type levels (Figure 2b). This raises the possibility that Vac1p may regulate the association of Vps45p with Pep12p via signaling from Vps21p and PI(3)P.

Together with our previous studies [1], we have shown that Vac1p binds many previously identified structural and regulatory factors required for the docking and fusion of Golgi-derived vesicles with the prevacuolar endosome (Figure 3); however, we cannot at present determine the order in which each factor binds to Vac1p. Interaction of Vps21p (on the vesicle) with Vac1p (on the endosome)

Figure 3



Multivalent Vac1p interactions regulate vesicle docking/fusion. The Vac1p motifs are shown above and the factors that bind Vac1p are shown immediately below. Vps9p is the GEF for Vps21p, causing the conversion of GDP-bound Vps21p to its GTP-bound form. Vps34p is the yeast PI 3-kinase, mediating conversion of PI to PI(3)P.

may play a role in binding/tethering vesicles to the endosome prior to SNARE interactions, in a manner similar to that which has been observed with Ypt1p and Uso1p at the ER-to-Golgi transport step [9]. Recently, it has become clear that t-SNARE and vesicular (v) SNARE pairing cannot be solely responsible for the specificity of vesicular trafficking [10]. Instead, other factors, including Rab GTPases and Sec1p family proteins, have been postulated to regulate vesicular docking and fusion [11]. A variation of this model is suggested from our studies of Golgi-to-endosome transport in the VPS pathway. Mutations in Vac1p or Pep12p result in the accumulation of undocked vesicles, apparently free in the cytoplasm, indicating that each of these proteins is required for the stable docking of vesicles [12,13]. In contrast, clusters of vesicles, possibly docked with the prevacuolar endosome, accumulate in *vps45* mutants, suggesting that docking does not require Vps45p [14]. We speculate that Vac1p may act as a multivalent adaptor protein with other stage-specific transport factors, such as Rab GTPases, Sec1p homologues and PI(3)P, to ensure the fidelity of vesicular transport reactions. The presence of PI(3)P and activation of Vps21p by Vps9p — resulting in the high-affinity binding of GTP-bound Vps21p to Vac1p — may allow progression of docking and/or fusion as long as other Vac1p ligands (for example, Pep12p and Vps45p) have been properly assembled.

Supplementary material

Additional methodological detail is published with this paper on the internet.

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References

1. Burd C, Peterson M, Cowles C, Emr S: A Novel Sec18p/NSF-dependent complex required for Golgi-to-endosome transport in yeast. *Mol Biol Cell* 1997, 8:1089-1104.
2. Burd C, Emr S: Phosphatidylinositol(3)phosphate signaling mediated by specific binding to RING FYVE domains. *Mol Cell* 1998, 2:157-162.
3. Webb GC, Hoedt M, Poole LJ, Jones EW: Genetic interactions between a pep7 mutation and the PEP12 and VPS45 genes: evidence for a novel SNARE component in transport between the ***Saccharomyces cerevisiae*** Golgi complex and endosome. *Genetics* 1997, 147:467-478.
4. Stack JH, Emr SD: Vps34p required for yeast vacuolar protein sorting is a multiple specificity kinase that exhibits both protein kinase and phosphatidylinositol-specific PI 3-kinase activities. *J Biol Chem* 1994, 269:31552-31562.
5. Horazdovsky B, Busch G, Emr S: VPS21 encodes a rab5-like GTP binding protein that is required for the sorting of yeast vacuolar proteins. *EMBO J* 1994, 13:1297-1309.
6. Mills IG, Jones AT, Clague MJ: Involvement of the endosomal autoantigen EEA1 in homotypic fusion of early endosomes. *Curr Biol* 1998, 8:881-884.
7. Simonsen A, Lippe R, Christoforidis S, Gaullier JM, Brech A, Callaghan J, *et al.*: EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. *Nature* 1998, 394:494-498.
8. Hamma H, Tall G, Horazdovsky B: Vps9p is a guanine nucleotide exchange factor involved in vesicle-mediated vacuolar protein transport. *J Biol Chem* 1999, in press.
9. Cao X, Ballew N, Barlow C: Initial docking of ER-derived vesicles requires Uso1p and Ypt1p but is independent of SNARE proteins. *EMBO J* 1998, 17:2156-2165.
10. Darsow T, Rieder SE, Emr SD: A multispecificity syntaxin homologue, Vam3p, essential for autophagic and biosynthetic protein transport to the vacuole. *J Cell Biol* 1997, 138:517-529.
11. Schimmoller F, Simon I, Pfeffer SR: Rab GTPases, directors of vesicle docking. *J Biol Chem* 1998, 273:22161-22164.
12. Webb GC, Zhang J, Garlow SJ, Riezman H, Jones EW: Pep7p provides a novel protein that functions in vesicle-mediated transport between the yeast Golgi and endosome. *Mol Biol Cell* 1997, 8:871-895.
13. Becherer KA, Rieder SE, Emr SD, Jones EW: Novel syntaxin homologue, Pep12p, required for the sorting of lumenal hydrolases to the lysosome-like vacuole in yeast. *Mol Biol Cell* 1996, 7:579-594.
14. Cowles C, Emr S, Horazdovsky B: Mutations in the VPS45 gene, a SEC1 homologue, result in vacuolar protein sorting defects and accumulation of membrane vesicles. *J Cell Sci* 1994, 107:3449-3459.
15. Horazdovsky BF, Emr SD: The VPS16 gene product associates with a sedimentable protein complex and is essential for vacuolar protein sorting in yeast. *J Biol Chem* 1993, 268:4953-4962.
16. Klionsky DJ, Emr SD: Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. *EMBO J* 1989, 8:2241-2250.
17. Grandi P, Doye V, Hurt EC: Purification of NSP1 reveals complex formation with 'GLFG' nucleoporins and a novel nuclear pore protein NIC96. *EMBO J* 1993, 12:3061-3071.

Supplementary material

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Supplementary materials and methods

CPY sorting analysis

The construction of *vac1* mutant alleles is described in [S1]. The GDP-locked form of *VPS21*, *vps21* (S21L), was cloned into a yeast CEN expression vector (pRS415). Four suppressor genes, full-length *VPS9*, *PEP12*, *VPS45* and *SEC19* (pRS423), were subcloned into 2μ expression vectors. All CPY-sorting analyses for this study were conducted in the SEY6210 *vac*1Δ::*URA3* yeast strain. Yeast cultures were radiolabeled using previously published procedures [S2]. Immunoprecipitations of CPY were performed using the method of Klionsky *et al*. [S3].

Two-hybrid analysis

The genes indicated in Table 1 were subcloned into either bait (pGBT9) or prey (pGADGH) vectors (Clontech). Constructs were introduced into a reporter strain (HF7c, Clontech), and tested for association by growth on plate media lacking histidine and supplemented by 1 mM 3-amino-2,4-triazole, an inhibitor of the *HIS3* gene product. Binding of bait and prey proteins causes transcription of the *HIS3* gene product and allows for growth on such media. After several days at 30°C, plates were scored for growth and placed into four groups, from +++ to –, depending on growth rate.

Co-precipitation analysis

A mutant form of *VPS21*, either *vps21* (Q66L) or *vps21* (S21L), was subcloned in-frame 3′ to a Protein-A domain in multicopy 2μ vectors (pRS424), and transformed into a wild-type strain (SEY6210). Two Protein-A domains were subcloned into the *Pac*1/*Bst*EII sites at the 3′ end of the full-length *VAC1* gene and the result subcloned into both CEN and multicopy 2μ yeast expression vectors (pRS415 and pRS425, respectively). Cultures containing the Protein-A-tagged constructs were grown to mid-log phase (0.5–0.8 OD_{600}). Approximately 200 OD_{600} units of cells were converted to spheroplasts and resuspended at 20 OD_{600}/ml in 150 mM KOAc, 5 mM MgOAc, 20 mM HEPES pH 8.0 with protease inhibitors. Cells were lysed by dounce homogenization, and lysates were centrifuged for 5 min at 300 × *g*. Digitonin was added to 1%, and lysates were shaken on ice for 10 min, and spun for 10 min at 13,000 × *g*. The supernatants were chromatographed on columns packed with 300 μl IgG–Sepharose (Pharmacia). The columns were washed three times with 1 ml 150 mM KOAc, 5 mM MgOAc, 20 mM HEPES pH 8.0, 0.05% digitonin, and once with 1 ml 5 mM ammonium acetate pH 5.0. Bound proteins were eluted with 1 ml 0.5 M acetic acid pH 3.4. Following TCA precipitation, 50 OD_{600} equivalents of each sample was subjected to SDS–PAGE and western blotting, with antisera raised against Vac1p [S1], Vps9p [S4] and Vps45p [S5]. Enhanced chemiluminescence was used to visualize immunoblots (Amersham). Quantitation of the Vps9p result was performed on a Macintosh computer using the public domain NIH Image program.

References

S1. Burd C, Peterson M, Cowles C, Emr S: A Novel Sec18p/NSF-dependent complex required for Golgi-to-endosome transport in yeast. *Mol Biol Cell* 1997, 8:1089-1104.

S2. Horazdovsky BF, Emr SD: The VPS16 gene product associates with a sedimentable protein complex and is essential for vacuolar protein sorting in yeast. *J Biol Chem* 1993, 268:4953-4962.

S3. Klionsky DJ, Emr SD: Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. *EMBO J* 1989, 8:2241-2250.

S4. Burd CG, Mustol PA, Schu PV, Emr SD: A yeast protein related to a mammalian Ras-binding protein, Vps9p, is required for localization of vacuolar proteins. *Mol Cell Biol* 1996, 16:2369-2377.

S5. Cowles C, Emr S, Horazdovsky B: Mutations in the VPS45 gene, a SEC1 homologue, result in vacuolar protein sorting defects and accumulation of membrane vesicles. *J Cell Sci* 1994, 107:3449-3459.